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pmoA Primers for Detection of Anaerobic Methanotrophs▽

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Published *pmoA* primers do not match the *pmoA* sequence of "*Candidatus* Methylomirabilis oxyfera," a bacterium that performs nitrite-dependent anaerobic methane oxidation. Therefore, new *pmoA* primers for the detection of "*Ca.* Methylomirabilis oxyfera"-like methanotrophs were developed and successfully tested on freshwater samples from different habitats. These primers expand existing molecular tools for the study of methanotrophs in the environment.

Aerobic methane oxidation is performed by several specialized groups of bacteria that are widely distributed in the environment (6). The first step of aerobic methane oxidation is the conversion of methane to methanol, catalyzed by either soluble or particulate methane monooxygenase (pMMO). The alpha subunit of pMMO, encoded by the *pmoA* gene, is highly conserved (5) and often used as a functional marker for analyzing methanotrophs in the environment (14). The first oligonucleotide primers targeting the *pmoA* gene, A189 and A682, have been used extensively in environmental studies (14). As the *pmoA* gene phylogeny is largely comparable to the 16S rRNA gene phylogeny (7, 12), *pmoA* primers provide a useful tool for obtaining simultaneous functional and taxonomic inventories of methanotrophs in the environment (10).

Recently, new *pmo*-like (*pxm*) genes in cultured, methanotrophic *Gammaproteobacteria* (21) and new groups of aerobic methane oxidizers with divergent *pmo* genes were discovered. Most noteworthy are the proteobacterial *Crenothrix polyspora* (19) and three acidophilic verrucomicrobial *Methylacidiphilum* species (16). The presence of a complete *pmo* gene cluster in the genome of the anaerobic, methanotrophic, nitrite-reducing bacterium "*Candidatus* Methylomirabilis oxyfera" (3) further expanded the array of *pmo* gene diversity. "*Ca.* Methylomirabilis oxyfera," a member of the uncultured NC10 phylum (17), forms a novel taxonomic group of bacterial methanotrophs (reviewed in reference 24). The presence of *pmo* genes in an anaerobe was explained by the unusual metabolism of "*Ca.* Methylomirabilis oxyfera": molecular oxygen was produced from nitric oxide, an intermediate of denitrification, and then used to oxidize methane via the complete aerobic pathway starting with pMMO (3).

It was not possible to amplify *pmoA* genes with the most commonly used forward primer A189 and the reverse primers A682 (7), mb661 (2), and A650 (1), even from "*Ca.* Methylomirabilis oxyfera" enrichment cultures. The alignment of the primers with the *pmoA* sequence of "*Ca.* Methylomirabilis oxyfera" revealed several critical mismatches especially with the reverse primers (Fig. 1), explaining the failure to obtain a PCR product. Based on this alignment, new *pmoA* primers were developed. We changed one nucleotide to a wobble base in the forward primer A189, resulting in a general degenerate primer, A189_b (Fig. 1), matching most methanotrophs in the GenBank database with the exception of the genus *Methylacidiphilum*. The reverse primer A682 differed in eight nucleotides from the *pmoA* sequence of "*Ca.* Methylomirabilis oxyfera." Therefore, the newly developed reverse primer designated cmo682 specifically targets "*Ca.* Methylomirabilis oxyfera"-like bacteria. Also, a second set of primers for a nested-PCR approach specific for nitrite-dependent anaerobic methane-oxidizing bacteria was developed based on this alignment. These primers were named cmo182 and cmo568 (Fig. 1) and were used in a nested approach after the amplification with A189_b and cmo682. The newly designed primers were first tested with DNA extracted from Ooijpolder drainage ditch sediment, which had previously been used to obtain an enrichment culture performing nitrite-dependent anaerobic methane oxidation (4). The same approach was then used to screen samples from several oxygen-limited freshwater habitats for the presence of "*Ca.* Methylomirabilis oxyfera"-like bacteria: an alpine peat bog (China), wastewater treatment plants (WWTP) (Netherlands), and contaminated aquifers (United States and Netherlands) (Table 1). There is circumstantial evidence for the occurrence of anaerobic methane oxidation in the contaminated aquifers (18, 22).

To extract DNA from the samples from Ooijpolder, Banisveld, Rotterdam, and Lichtenvoorde (Table 1), biomass was collected by centrifugation. The pellets were resus-

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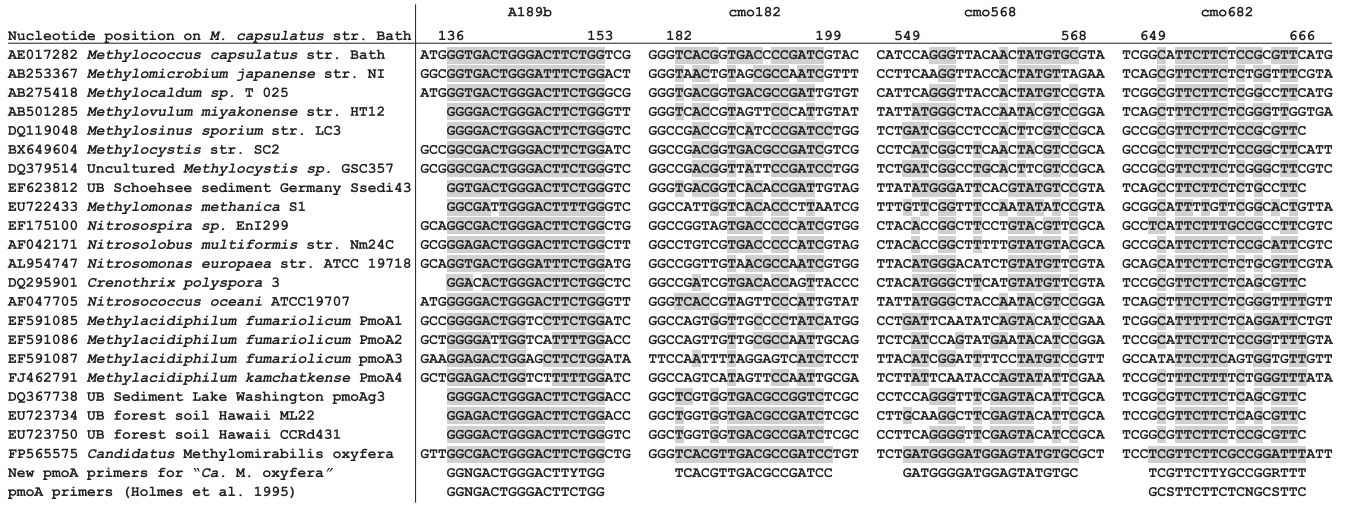

FIG. 1. Alignment using MEGA4 of newly developed primers for the alpha subunit of particulate methane monooxygenase (*pmoA*) of "*Candidatus* Methylomirabilis oxyfera" and nucleotide sequences encoding the *pmoA* genes of different methanotrophic *Gammaproteobacteria* (GenBank accession no. AE017282, AB253367, AB275418, AB501285, DQ295901), methanotrophic *Alphaproteobacteria* (DQ119048, BX649604, DQ379514, EF623812), and *Verrucomicrobia* (EF591085, EF591086, EF591087, FJ462791); different ammonia monooxygenase (*amoA*) sequences of nitrifying *Betaproteobacteria* (EF175100, AF042171, AL954747); *amoA* sequences of nitrifying *Gammaproteobacteria* (AF047705); *pxmA* sequences of methanotrophic *Gammaproteobacteria* (EU722433); and *pmoA* sequences of different uncultured methanothrophs of unknown affiliation (DQ367738, EU723734, EU723750) (20). Identical nucleotides are shaded in gray. cmo568 and cmo682 are shown as the reverse complementary sequence.

pended in 120 mM sodium phosphate buffer, glass beads (~0.3 g, 0.25-mm diameter) were added, and bead beating (Retsch MM301) was performed for 60 s at 30 Hz. DNA was extracted and purified according to reference 13. For the nitrogen-contaminated Cape Cod aquifer (15), 2 liters of groundwater from a depth of 8.9 m (relative to mean sea level) at sampling site F168 was filtered using a Sterivex cartridge filter (0.2-μm membranes; Millipore). The membrane filter was used for DNA extraction. DNA from Cape Cod and Zoige soil (0.25 g) was extracted using the PowerSoil DNA isolation kit (MO BIO Laboratories, Inc.) according to the manufacturer's protocol.

To amplify the *pmoA* gene fragment, the primers A189_b and cmo682 were used for the first PCR, and cmo182 and cmo568 were used for the second PCR. For both PCRs, thermal cycling was performed with an initial melting step for 4 min at 94°C, followed by 35 cycles of denaturation at 94°C for 1 min, annealing using a temperature gradient from 50 to 60°C (Zoige sample, 53 to 63°C) for 1 min, and elongation at 72°C for 1.5 min. Finally, an elongation step at 72°C for 10 min was performed. After PCR, amplicons of all annealing temperatures were pooled to minimize random polymerase errors and the effects of primer mismatches. All PCRs were performed with PerfeCTa SYBR Green FastMix (Quanta BioSciences, Inc., Gaithersburg, MD).

PCRs with the primers A189_b and cmo682 on DNA from Ooijpolder sediment yielded faint and multiple PCR bands, whereas the nested PCR using the pooled amplicons of the first PCR with primers cmo182 and cmo568 resulted in a strong and single band on the gel of the correct size (389 bp). The PCR product mix was ligated into the pGEM-T Easy cloning vector and cloned according to the manufacturer's instructions (Promega). Plasmids were isolated from randomly selected clones for each PCR product with the GeneJET miniprep kit (Fermentas, Lithuania). The plasmids were sequenced using the M13 forward primer. Sequences were checked for quality with Chromas Lite 2.01, erroneous sequences were removed, and BLAST search analysis was performed (http://www.ncbi.nlm.nih.gov/Blast.cgi). Sequences matching *pmoA* were aligned to a data set of different *pmoA*, *pxmA*, and *amoA* sequences using the MEGA4 software package and the Clustal W algorithm (20). A phylogenetic tree with the *pmoA* sequences of *Methylacidiphilum* as an outgroup was calculated with the neighbor-joining method, and tree to-

TABLE 1. Overview of sampling locations

Site abbreviation	Sampling location	Geographic coordinate	Environment[a]	Reference
Ooijpolder	Nijmegen, Netherlands	51°50′N, 5°54′E	Ditch	Ettwig et al. (4)
Zoige	Tibetan plateau, China	33°56′N, 102°52′E	Peat bog	Zhang et al. (25)
Cape Cod	Massachusetts, United States	41°41′N, 70°17′W	Aquifer, well F168	Smith et al. (18); Miller and Smith (15)
Banisveld	Oirschot, Netherlands	51°33′N, 5°17′E	Aquifer, 3.2-m depth	van Breukelen and Griffioen (22)
Lichtenvoorde	Lichtenvoorde, Netherlands	51°59′N, 6°34′E	WWTP	WWTP Lichtenvoorde[b]
Rotterdam	Rotterdam, Netherlands	51°55′N, 4°28′E	WWTP	van der Star et al. (23)

[a] WWTP, wastewater treatment plant.
[b] http://www.waterstromen.nl/onze_activiteiten/afvalwaterbehandeling/afvalwaterzuivering_royal_dutch_tanneries_lichtenvoorde.aspx.

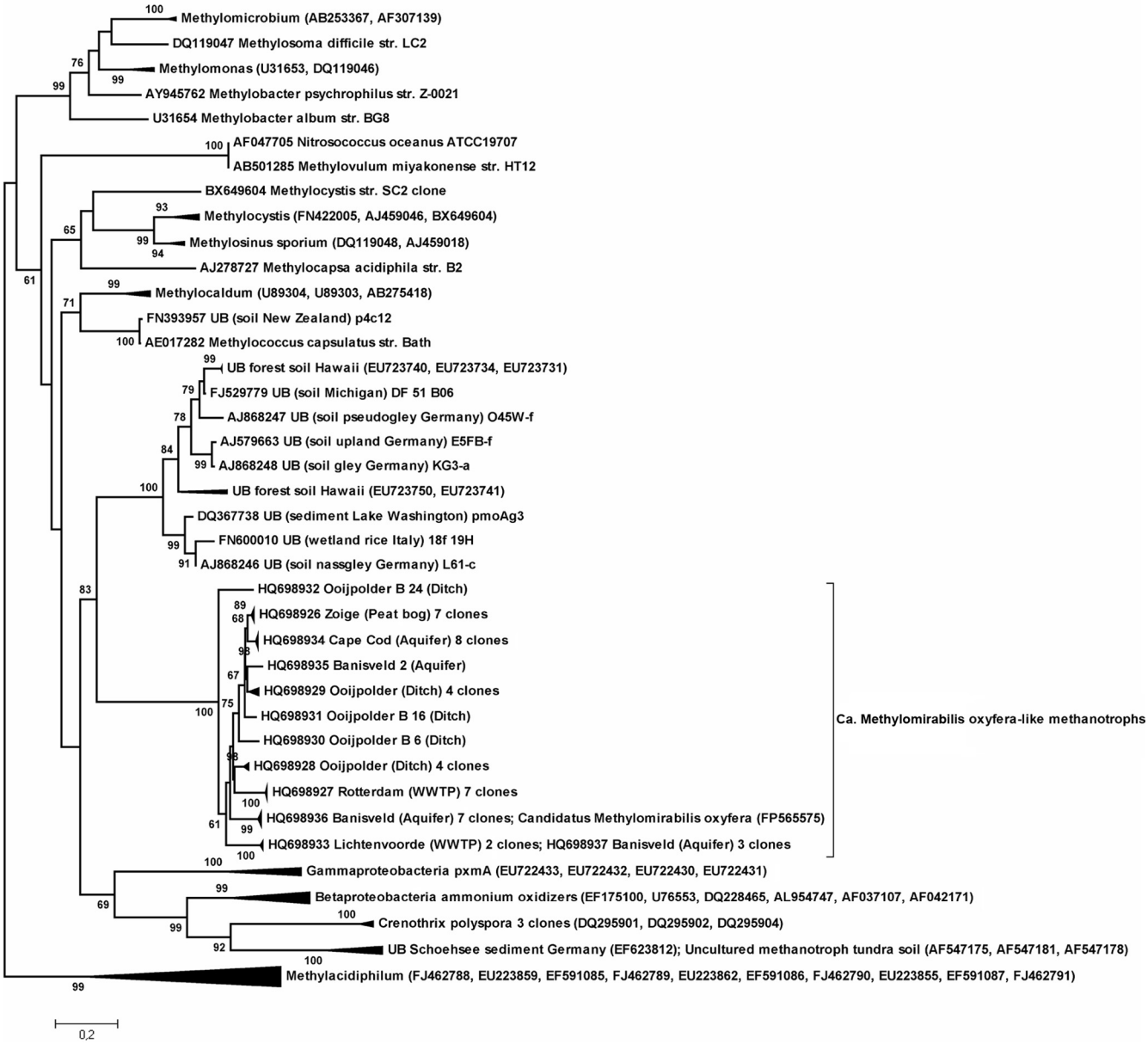

FIG. 2. Phylogenetic tree of *amoA*, *pmoA*, and *pxmA* nucleotide sequences, including the sequences obtained in this study with *Methylacidiphilum* as the outgroup. The tree was calculated using the neighbor-joining method. Bootstrap support values greater than 60% (n = 1,000) are indicated at the nodes.

pology robustness was tested by bootstrap analysis of 1,000 replicates (Fig. 2).

Similar to the results with the Ooijpolder samples, the direct PCR on the different environmental samples using the primer combination A189_b and cmo682 yielded multiple PCR products. Therefore, the same nested approach was applied as described above. From all the different environments analyzed (Table 1), *pmoA* sequences closely related to "*Ca.* Methylomirabilis oxyfera" (at least 85.5% nucleotide identity and 92% protein identity) were retrieved, most of which cluster according to location (Fig. 2). Together they form a distinct group affiliated with the *pmoA* genes of aerobic methanotrophs (Fig. 2). The closest related sequences obtained with PCR using the conventional *pmoA* primers (8, 9, 11) were not targeted by the new primers (Fig. 1). All newly obtained sequences share the insertion of a proline at position 131 (3), a distinct feature compared to the sequences of known methane or ammonium monooxygenases. The fact that they are not very divergent from each other may reflect functional conservation relative to the oxygenic pathway.

This study provides a rapid and robust method for the amplification of NC10 phylum *pmoA* genes and enables the screening of different environments.

Nucleotide sequence accession numbers. Representative sequences from each location were submitted to GenBank under accession numbers HQ698926 to HQ698937.

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REFERENCES

1. **Bourne, D. G., I. R. McDonald, and J. C. Murrell.** 2001. Comparison of *pmoA* PCR primer sets as tools for investigating methanotroph diversity in three Danish soils. Appl. Environ. Microbiol. **67:**3802–3809.
2. **Costello, A. M., and M. E. Lidstrom.** 1999. Molecular characterization of functional and phylogenetic genes from natural populations of methanotrophs in lake sediments. Appl. Environ. Microbiol. **65:**5066–5074.
3. **Ettwig, K. F., et al.** 2010. Nitrite-driven anaerobic methane oxidation by oxygenic bacteria. Nature **464:**543–548.
4. **Ettwig, K. F., T. van Alen, K. T. van de Pas-Schoonen, M. S. M. Jetten, and M. Strous.** 2009. Enrichment and molecular detection of denitrifying methanotrophic bacteria of the NC10 phylum. Appl. Environ. Microbiol. **75:**3656–3662.
5. **Hakemian, A. S., and A. C. Rosenzweig.** 2007. The biochemistry of methane oxidation. Annu. Rev. Biochem. **76:**223–241.
6. **Hanson, R. S., and T. E. Hanson.** 1996. Methanotrophic bacteria. Microbiol. Rev. **60:**439–471.
7. **Holmes, A. J., A. Costello, M. E. Lidstrom, and J. C. Murrell.** 1995. Evidence that particulate methane monooxygenase and ammonia monooxygenase may be evolutionarily related. FEMS Microbiol. Lett. **132:**203–208.
8. **Kalyuzhnaya, M. G., et al.** 2006. Fluorescence in situ hybridization-flow cytometry-cell sorting-based method for separation and enrichment of type I and type II methanotroph populations. Appl. Environ. Microbiol. **72:**4293–4301.
9. **King, G. M., and K. Nanba.** 2008. Distribution of atmospheric methane oxidation and methanotrophic communities on Hawaiian volcanic deposits and soils. Microbes Environ. **23:**326–330.
10. **Kip, N., et al.** 2010. Global prevalence of methane oxidation by symbiotic bacteria in peat-moss ecosystems. Nat. Geosci. **3:**617–621.
11. **Knief, C., S. Kolb, P. L. E. Bodelier, A. Lipski, and P. F. Dunfield.** 2006. The active methanotrophic community in hydromorphic soils changes in response to changing methane concentration. Environ. Microbiol. **8:**321–333.
12. **Kolb, S., C. Knief, S. Stubner, and R. Conrad.** 2003. Quantitative detection of methanotrophs in soil by novel *pmoA*-targeted real-time PCR assays. Appl. Environ. Microbiol. **69:**2423–2429.
13. **Kowalchuk, G. A., F. J. de Bruijn, I. M. Head, A. D. Akkermans, and J. D. van Elsas.** 2004. Molecular microbial ecology manual (MMEM), 2nd ed., vol. 1. Kluwer Academic Publishing, London, United Kingdom.
14. **McDonald, I. R., L. Bodrossy, Y. Chen, and J. C. Murrell.** 2008. Molecular ecology techniques for the study of aerobic methanotrophs. Appl. Environ. Microbiol. **74:**1305–1315.
15. **Miller, D. N., and R. L. Smith.** 2009. Microbial characterization of nitrification in a shallow, nitrogen-contaminated aquifer, Cape Cod, Massachusetts and detection of a novel cluster associated with nitrifying *Betaproteobacteria*. J. Contam. Hydrol. **103:**182–193.
16. **Op den Camp, H. J. M., et al.** 2009. Environmental, genomic and taxonomic perspectives on methanotrophic *Verrucomicrobia*. Environ. Microbiol. Rep. **1:**293–306.
17. **Raghoebarsing, A. A., et al.** 2006. A microbial consortium couples anaerobic methane oxidation to denitrification. Nature **440:**918–921.
18. **Smith, R. L., B. L. Howes, and S. P. Garabedian.** 1991. In situ measurement of methane oxidation in groundwater by using natural-gradient tracer tests. Appl. Environ. Microbiol. **57:**1997–2004.
19. **Stoecker, K., et al.** 2006. Cohn's Crenothrix is a filamentous methane oxidizer with an unusual methane monooxygenase. Proc. Natl. Acad. Sci. U. S. A. **103:**2363–2367.
20. **Tamura, K., J. Dudley, M. Nei, and S. Kumar.** 2007. MEGA4: Molecular Evolutionary Genetics Analysis (MEGA) software version 4.0. Mol. Biol. Evol. **24:**1596–1599.
21. **Tavormina, P. L., V. J. Orphan, M. G. Kalyuzhnaya, M. S. M. Jetten, and M. G. Klotz.** 2010. A novel family of functional operons encoding methane/ammonia monooxygenase-related proteins in gammaproteobacterial methanotrophs. Environ. Microbiol. Rep. **3:**91–100.
22. **van Breukelen, B. M., and J. Griffioen.** 2004. Biogeochemical processes at the fringe of a landfill leachate pollution plume: potential for dissolved organic carbon, Fe(II), Mn(II), NH_4, and CH_4 oxidation. J. Contam. Hydrol. **73:**181–205.
23. **van der Star, W. R., et al.** 2007. Startup of reactors for anoxic ammonium oxidation: experiences from the first full-scale anammox reactor in Rotterdam. Water Res. **41:**4149–4163.
24. **Wu, M. L., et al.** 2011. A new intra-aerobic metabolism in the nitrite-dependent anaerobic methane-oxidizing bacterium "*Candidatus* Methylomirabilis oxyfera." Biochem. Soc. Trans. **39:**243–248.
25. **Zhang, G. S., et al.** 2008. Methanogen community in Zoige wetland of Tibetan plateau and phenotypic characterization of a dominant uncultured methanogen cluster ZC-I. Environ. Microbiol. **10:**1850–1860.